Original Article

Solubility Enhancement of Embelin by Complexation with Beta Cyclodextrin

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ABSTRACT

Introduction: Embelin, a phytoconstituent obtained from *Embelia ribes* of the Myrsinaceae family, has anti-cancer, anti-inflammatory, anti-bacterial, anti-fertility, analgesic, antidiabetic, anti-depressant and wound healing activities. It is hydrophobic in nature leading to low bioavailability. **Aim:** The present study aims to improve the water solubility and rate of dissolution of Embelin by complexation with β-cyclodextrin. **Methods:** Inclusion complexes were prepared by physical mixture, kneading and co-precipitation methods. Characterization of complexes was carried out by Fourier-Transform Infrared (FT-IR) spectroscopy and *in vitro* dissolution study. Differential scanning calorimetry (DSC) and Scanning electron microscopy (SEM) was used to analyze the prepared complexes prepared by the co-precipitation method. Antimicrobial studies of complexes against *Staphylococcus aureus* and *Escherichia coli* were carried out by colony counting method. **Results:** Phase solubility study showed Embelin forms complex with β-cyclodextrin in the ratio 1:2. FT-IR studies of complexes confirmed Embelin forms complex with β-cyclodextrin. DSC and SEM also confirmed the formation of a complex of Embelin with β-cyclodextrin. *In vitro* dissolution studies showed that the time to release 50 % (t50) of Embelin was in the order 15 min, 30 min, 60 min for complexes prepared by co-precipitation, kneading method and physical mixture respectively. Complexes prepared by the coprecipitation method showed 2 log reductions in the number of *S. aureus* and 1 log reduction in the number of *E. coli* in comparison with Embelin. **Conclusion:** Complexes of Embelin prepared by co-precipitation method resulted in largest percent drug content, enhanced aqueous solubility and antibacterial activity.

Key words: Embelin, β-cyclodextrin, Inclusion complexes, Solubility, Co-precipitation method.

INTRODUCTION

Potential health benefits and less toxicity of natural products made them the first choice for the search for new drugs.[1] Embelin (Figure 1) is a phytoconstituents obtained from the plant *Embelia ribes* from the Myrsinaceae family.[2,3] It has anticancer,[4] anti-inflammatory,[5] anti-bacterial,[6] anti-fertility,[7] analgesic,[8] antidiabetic,[9] anti-depressant,[10] and wound healing activities.[11] Molecular weight of Embelin is 294.391 g/ mol and melting point is 142.5°C. It is lipophilic in nature with a log P of 4.34.[12] Embelin has low solubility[13] in water (0.2-0.3 mg/ml)[14] and less bioavailability (30±11%).[2] It is a 2,5-dihydroxy-3-undecyl-1, 4-benzoquinone.[7]

Cyclodextrins (CDs) are obtained from the enzymatic degradation of starch.[15] They can form complexes with drug molecules which is favored by cyclodextrin's unique ring structure made by binding of glucose units. Such complexes can improve the physicochemical properties of drugs without changes in their molecular level[16] rendering the name 'enabling pharmaceutical ingredients' for cyclodextrins.[17] The α, ß and γ-CD cyclodextrins are composed of six, seven and eight D-(+) -glucopyranose units[18] respectively. Present study uses β-cyclodextrin which is cheap, biocompatible, possesses adequate cavity

Submission Date: 10-08-2020;
Revision Date: 27-02-2021;
Accepted Date: 12-04-2021

DOI: 10.5530/ijper.55.2s.112

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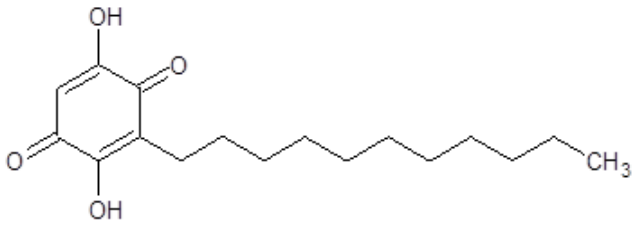

Figure 1: Structure of Embelin.

size and effective drug complexation.[19] Drug permeation through biological membranes can be enhanced by the formation of water-soluble inclusion complexes.[20] The amount of CD has to be optimum for maximum permeation. Kneading and co-precipitation are the two most commonly used methods for the preparation of inclusion complexes. CDs have the potential for the delivery of poorly soluble drugs to the body. So, they can be used as a tool to deliver active pharmacophores that lack the required physicochemical attributes for optimum bioavailability to the body.[21] Drugs encapsulated in particulate systems in the presence of CDs have shown improved permeability and bioavailability.[22] Layered tablets based on CDs has been developed for non-steroidal anti-inflammatory drugs.[23] CDs can modify the release of drugs from different drug delivery systems.[24] Cyclodextrins based formulations for improving patient compliance have been reported.[25]

In order to increase its solubility and thereby bioavailability, Embelin is complexed with cyclodextrin to form inclusion complexes. Evaluation of prepared complexes can confirm the formation of complexes. It also reveals the extent of solubility enhancement of Embelin and modifications in its antimicrobial activity.

MATERIALS

Embelin (YUCCA enterprises with a certificate of analysis having the purity of 98%, Mumbai, Maharashtra), β-cyclodextrin (Chemdyes Co-operation, Rajkot, Gujarat), Mc Farland turbidity standard (Chemdyes Co-operation, Rajkot, Gujarat), Nutrient broth (HiMedia laboratories private Ltd, Bengaluru, Karnataka), Agar-agar (Research lab fine chem industries, Mumbai, Maharashtra), Sodium hydroxide (Nice chemicals, Kochi), Potassium dihydrogen phosphate (Nice chemicals, Kochi), Methanol (Nice chemicals, Kochi), Whatman filter paper (0.45µm), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538P) (NCIM, CSIR, Pune, Maharashtra).

METHODS

Phase solubility studies

Higuchi and Connors method was used to carry out phase solubility studies.[26] Excess amounts of Embelin was added to vials containing β-cyclodextrin solution (5ml) in a mixture of methanol and water (25/75 v/v) at a different concentration ranging from $0.1x10^{-2}$ M to $1.0x10^{-2}$ M. The vials were shaken at 32°C for 48 h. Centrifuge the sample for 10 min at 3000 rpm. The resulting solution was then filtered through Whatman filter paper size (0.45µm) and analyzed by UV-Visible spectrophotometer at 324.6nm.[27,28]

Preparation of complexesPhysical mixture

The required molar ratio (1:2) quantities of the Embelin and β-cyclodextrin were accurately weighed and mixed for 45 min by trituration in a mortar. The mixture was passed through sieve No: 44 and stored in airtight containers.[29]

Kneading method

Embelin and β-cyclodextrin were weighed in the ratio of 1:2. Added small quantities of water and methanol (1:2) mixture till a homogeneous paste was obtained. To this Embelin powder was added in portions and kneading was continued for one hour. Paste consistency was maintained by adding a methanol-water mixture. A hot air oven was used to dry the prepared paste at 45°-50°C for 24 h. Product was passed through sieve No: 44 and stored in airtight containers.[29,30]

Co-precipitation method

Weighed Embelin and β-cyclodextrin in the molar ratio (1:2). Dissolved the weighed ingredients in methanol: water. The Embelin solution was added drop-wise into cyclodextrin solution. Stirring was continued for 6 h in a mechanical shaker. The product was dried at 45-50°C for 48 h and stored in airtight containers.[29]

The percentage yield[30] of the prepared complex was also determined using the formula

$$\text{Percentage yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \text{ X } 100$$

Fourier-transform infrared spectroscopy (FT-IR)

FT-IR spectrum of standard Embelin and inclusion complexes was obtained by using FT-IR Spectrophotometer, Nicolet–iS5 [Id3 ATR-Ge], Thermo scientific, USA. The scans were obtained from 4000 to 400 cm^{-1} at a resolution of 1 cm.

Differential scanning calorimetry (DSC)

DSC analyzer (TA INSTRUMENTS Q 20 USA) was used to perform DSC analysis of Embelin, β-cyclodextrin and inclusion complexes prepared by co-precipitation method. A sample (5 mg) was sealed in an aluminum pan and subjected to heating at a rate of 10°C /min from 25–250°C under a nitrogen atmosphere.

Scanning electron microscopy (SEM)

Scanning electron microscope (SEM-JOEL Instruments, JSM-7610F, Japan) was used for studying surface morphology of inclusion complex prepared by co-precipitation method. After mounting the samples on the aluminum stub, they are dried at 37°C. Then it is coated with a thin gold-ion layer (3nm) using a sputter coated unit. An acceleration voltage of 15 kV was applied and the micrographs were examined at ×1000 and ×5000, x10000, x20000 magnifications.

Percent drug content estimation

Weighed complex equivalent to 5 mg drug was transferred to 100 ml standard flask. Made up the solution with phosphate buffer of pH 7.4 and after UV absorbance was measured at 339.2 nm using UV-Visible spectrophotometer.[31]

In vitro dissolution studies

In vitro dissolution studies were carried out for pure Embelin and the inclusion of CD complexes to compare the solubility of complexes prepared by different methods. US Pharmacopeia Type I dissolution test apparatus was used for the study. CD complexes equivalent to contain 10 mg Embelin were placed in dissolution vessels containing 900 ml of phosphate buffer of pH 7.4 kept at 37± 0.5°C and stirred at 50 rpm. Samples were collected periodically and replaced with a fresh dissolution medium. 10 ml was withdrawn and filtered through Whatman filter paper. Absorbance was read at 339.2 nm against a blank.[30-32]

Antibacterial studies

The colony counting method was used for the evaluation of antibacterial activity against *E. coli* and *S. aureus*. *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538P) cells were grown 24 h on a shaker at 100 rpm and 37°C. Using 0.5 Mc Farland turbidity standard (1–2x10^8 CFU/ml) as standard, bacterial suspensions were prepared in phosphate buffer solution (PBS) to get a density of 1x10^8 colony forming units (CFU)/ml. 100 mg of UV sterilized Embelin and inclusion complexes were then added to the bacterial suspension. The media was incubated at 37°C for 24 h. Bacterial culture was diluted in PBS. Nutrient agar plates were spread with this culture and incubated at 37°C overnight. Counted the CFU/ml. The experiments were performed in duplicate and the results are given as mean ± standard deviation. The following equation was used to calculate the antibacterial activity of complexes

Percent Antibacterial activity = (X-Y)/X*100

Where X is the number of colonies (CFU/ml) in the control group, Y is the number of colonies after the Embelin/CD complex was added.[33]

RESULTS

Phase solubility studies

Using Higuchi and Connors method[21] it was found that Embelin forms complex β-cyclodextrin within the ratio 1:2. The results are given in Table 1. The phase solubility diagram is shown in Figure 2.

The percentage yield of the prepared inclusion complexes was 96.35%, 67% and 82% for physical mixture, kneading method and co-precipitation method respectively.

Fourier-transform infrared spectroscopy (FT-IR)

The characteristic peak of Embelin (Figure 7) was observed at 3295 cm^-, 2857 cm^- and 2922 cm^-, 1680 cm^-, 1117 cm^- and 1320 cm^- due to O-H stretching, C-H stretching vibration, presence of alkene and alcoholic C-O bond respectively. The peaks of β-cyclodextrin (Figure 4) were seen at 3301 cm^-, 1619 cm^-, 1490 cm^-, 1320 cm^- and 1360 cm^-, 1150 cm^-, 1028 cm^- due to O-H stretching, an amide bond, O-H bending, α-CH_2 bending, O-H bending C-C-C bonding respectively. The peaks for inclusion complexes prepared by physical

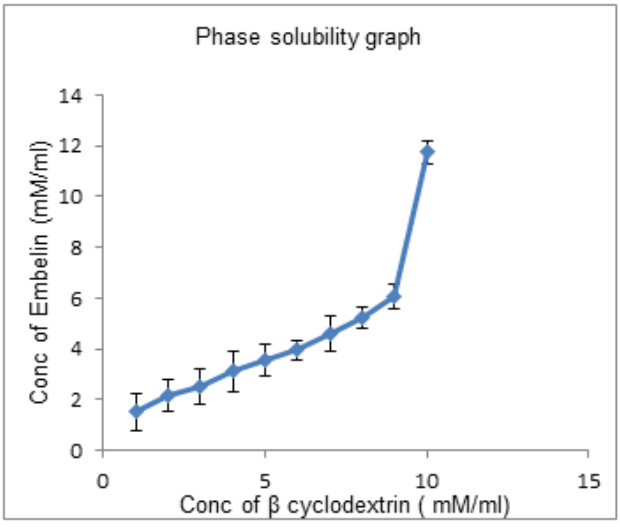

Figure 2: Phase solubility graph.

Table 1: Phase solubility studies.	
Concentration of β cyclodextrin (mM/ml)	**Concentration of Embelin(mM/ml)**
1	1.514 ± 0.722
2	2.157 ± 0.645
3	2.533 ± 0.708
4	3.117 ± 0.788
5	3.588 ± 0.633
6	3.956 ± 0.384
7	4.606 ± 0.712
8	5.236 ± 0.438
9	6.083 ± 0.486
10	11.741 ± 0.456

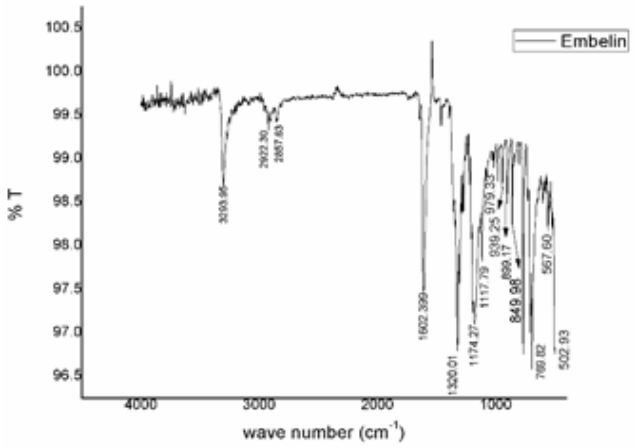

Figure 3: FT-IR spectrum of Embelin.

mixtures, kneading method and co-precipitation method were depicted in Figure 3, 5, 6 respectively.

Differential scanning calorimetric (DSC) analysis

The endothermic peaks for Embelin (Figure 8) are observed at 142. 19°C, 86. 87°C and 78. 9°C. The peak is observed at 110.7°C for β-cyclodextrin (Figure 9). Complexes prepared by the co-precipitation method show peaks at 218.5°C, 101.2°C (Figure 10).

Scanning electron microscopic (SEM) analysis

SEM image of inclusion complexes prepared by the co-precipitation method is shown in Figure 11.

Percent drug content

The percent drug content of prepared inclusion complexes was 48.69 ± 0.774% for physical mixture, 84.90 ±0.486% for kneading method and 96.14 ± 0.341% for the co-precipitation method.

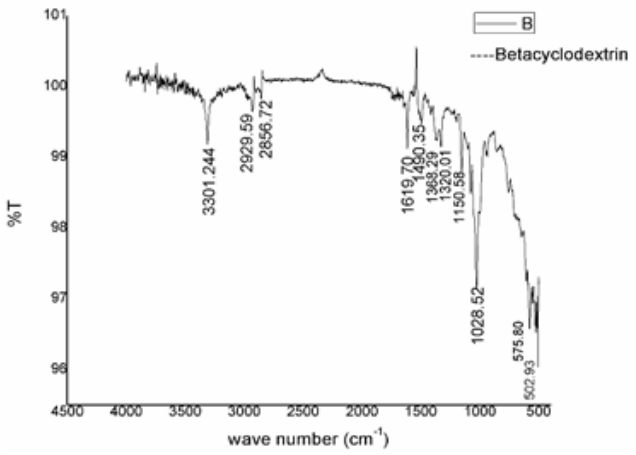

Figure 4: FT-IR spectrum of β cyclodextrin.

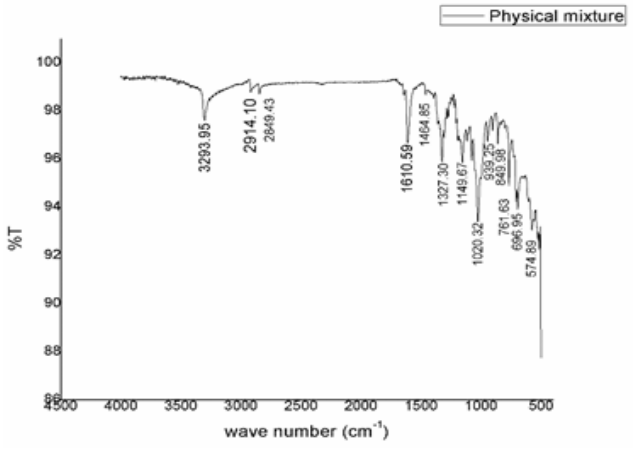

Figure 5: FT-IR spectrum of physical mixture.

In vitro dissolution study

The percent release of Embelin from complexes prepared by physical mixture, kneading method and co-precipitation method is shown in Table 2 and plotted in the graph Figure 12. Results for pure Embelin are also shown.

Antibacterial activity

The results of antibacterial activity are given as a log CFU graph in the Figure 13 and percent antibacterial activity is depicted in Table 3.

DISCUSSION

When the solubility of the Embelin increases with increasing β-cyclodextrin concentration A-type phase solubility profiles are obtained.[25] Phase solubility profiles are A_p- the type which indicates solubility of Embelin increases with an increase in β- cyclodextrin concentration and Embelin form a complex with cyclodextrin in the ratio 1: 2.

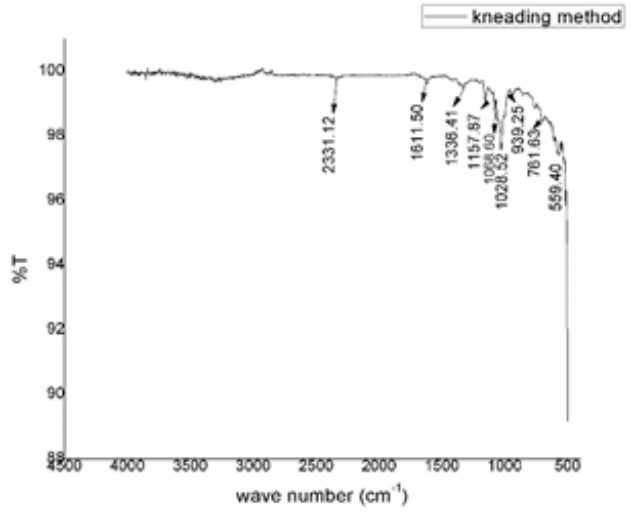

Figure 6: FT-IR spectrum of complexes prepared by kneading method.

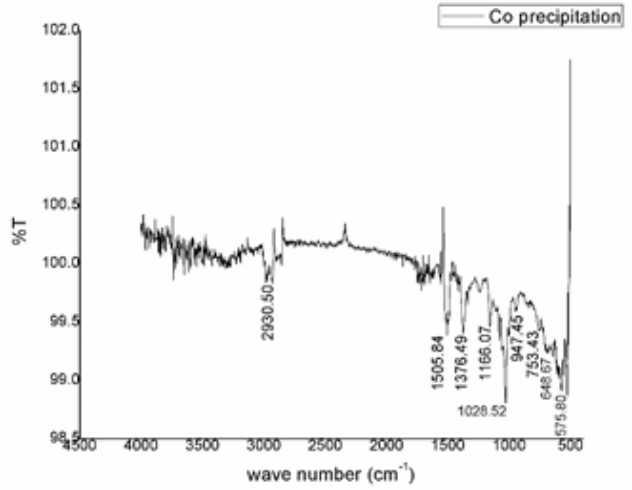

Figure 7: FT-IR spectrum of complexes prepared by co-precipitation method.

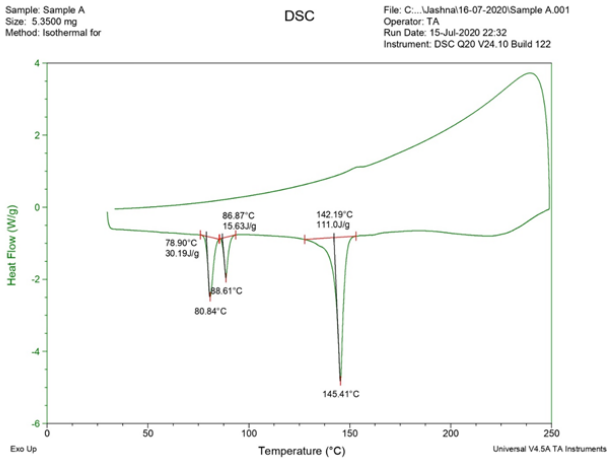

Figure 8: DSC of Embelin.

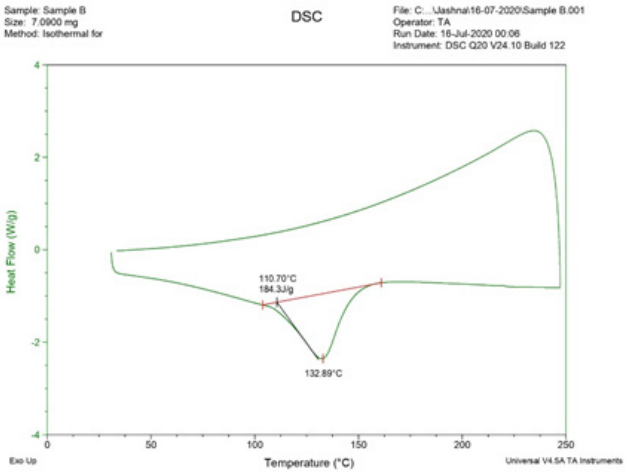

Figure 9: DSC of cyclodextrin.

Pure Embelin shows peaks at 3290 cm^{-1} due to alcoholic O-H stretching, 2857 cm^{-1} and 2922 cm^{-1} due to stretching vibration of C-H bond, 1680 cm^{-1} by the presence of alkene and 1117 cm^{-1},1174 cm^{-1}, 1320 cm^{-1} due to alcoholic C-O bond[34,35] (Figure 7). β-cyclodextrin shows peaks at 3301 cm^{-1}, 1619 cm^{-1}, 1490 cm^{-1}, 1320 cm^{-1}, 1360 cm^{-1}, 1150 cm^{-1}, 1028 cm^{-1} due to stretching of O-H bond, an amide bond, in-plane O-H bending, α-CH_2 bending, O-H bending, C-C-C bending respectively[36,37] (Figure 4). Physical mixture shows spectra which were superimposition of spectra of Embelin and β- cyclodextrin with minor alterations indicating the absence of interaction[38] (Figure 3). Complexes prepared by kneading method showed a shift in 2331 cm^{-1}, 1028 cm^{-1}, 1619 cm^{-1} to 1611 cm^{-1}, 1320 cm^{-1} to 1336 cm^{-1}, 1150 cm^{-1} to 1157 cm^{-1} which shows the peaks are more likely towards the peak of β- cyclodextrin (Figure 5). Complexes prepared by co-precipitation method showed shift in 2830 cm^{-1} to 2930 cm^{-1}, 1602 cm^{-1} to 1505 cm^{-1}, 1320 cm^{-1} to 1376 cm^{-1}, 1117 cm^{-1} to 1166 cm^{-1} (Figure 6). In comparison with peaks of Embelin, spectra of complexes showed changes in the position of peaks and a considerable decrease in intensity. This indicates an encapsulation of the benzoquinone ring of Embelin in β- cyclodextrin cavity. The spectrum of the physical mixture was similar to that of pure Embelin. Spectra of complexes prepared by kneading and co-precipitation method showed similarity to spectra of β- cyclodextrin. All absorption peaks of cyclodextrin polymer can be found but all characteristic peaks of Embelin almost disappear. Only a faint O-H and C-O vibration were observed at the 3200 cm^{-1} and 1600 cm^{-1} range, which provides substantial evidence of the formation of the Embelin/ β cyclodextrin inclusion complex.

Table 2: *In vitro* drug release study.				
Time (min)	**Embelin**	**Physical mixture**	**Kneading method**	**Co-precipitation method**
0	0	0	0	0
5	23.023±2.22	30.412±2.02	39.049±4.02	42.116±3.54
10	26.189±3.28	33.671±1.33	48.421±3.88	49.773±3.63
15	27.264±3.33	40.188±2.41	48.421±3.56	53.602±3.75
30	30.434±2.24	44.532±2.51	53.107±3.21	61.259±3.21
45	32.561±2.22	49.963±2.21	59.354±3.54	67.640±4.11
60	33.643±1.91	53.222±3.35	64.040±3.63	71.469±4.21
120	33.680±3.02	59.739±2.45	71.850±2.92	77.850±3.53
180	34.764±2.31	64.083±3.04	76.536±3.75	84.231±3.89
240	35.848±3.05	65.169±2.99	78.098±3.41	89.336±3.91
300	39.028±2.35	69.514±2.63	85.908±3.22	96.994±3.14

Min: minutes

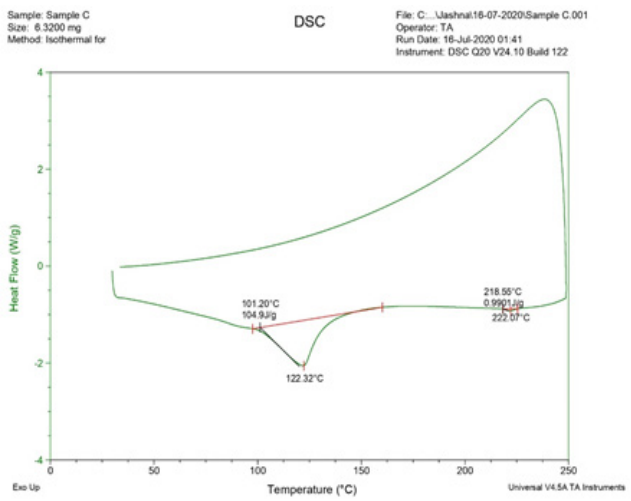

Figure 10: DSC of co-precipitation.

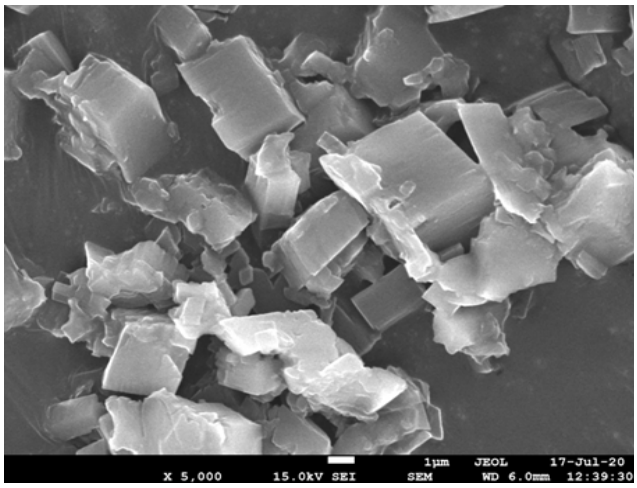

Figure 11: SEM image of complexes prepared by co-precipitation method.

DSC is a thermal analysis technique that processes the temperature and heat flow related through transitions in materials as a function of temperature and time.

The DSC of Embelin exhibited three endothermic peaks at 86.87°C, 78.90°C and 142.19°C (Figure 8) resulting from loss of water, dehydration of the compound and melting point. β cyclodextrin shows a characteristic endothermic peak at 110.70°C (Figure 9) which is its melting point. Complexes prepared by the co-precipitation method show peak at 101.20°C (Figure 10). The characteristic endothermic peak of Embelin corresponding to the melting point has disappeared which indicates that the Embelin is complexed or encapsulated in the hydrophobic cavity of β cyclodextrin. SEM image (Figure 11) of complexes prepared by co-precipitation shows smooth surface and regular shape. This may be due to the complication of Embelin in β- cyclodextrin which is again confirmed by FT-IR and DSC analysis.

The dissolution study of pure Embelin, physical mixture and inclusion complexes were carried out. The inclusion complexes of Embelin with β cyclodextrin could produce a considerable enhancement in the solubility of Embelin. Time to release 50 % (t_{50}) of Embelin was in the order 15 min, 30 min and 60 min for complexes prepared by co-precipitation, kneading method and physical mixture respectively (Figure 12). Complexes prepared by co-precipitation method showed t_{90} (Time to release 90 %) value for Embelin as four hours. The cumulative drug release by physical mixture, complexes prepared by kneading method and pure Embelin at the fourth hour was 70 %, 86 % and 39 % respectively. It is evident from the results that the degree of improvement in the dissolution rate depends upon the method of preparation. Complex formation at the molecular level

Table 3: Percent Antibacterial activity.		
Organisms	***E. coli***	***S. aureus***
Physical mixture	30.43±0.2	52.0±0.07
Kneading method	47.82±0.1	87.2±0.2
Co-precipitation method	96.95±0.01	97.6±0.02

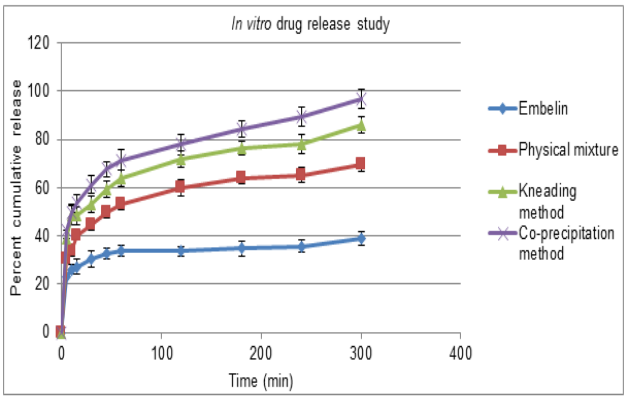

Figure 12: *In vitro* drug release study.

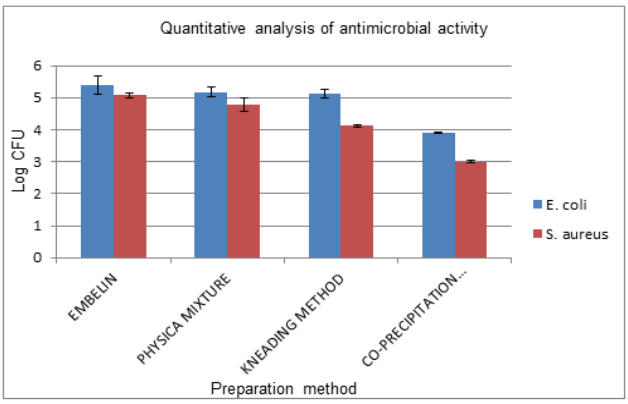

Figure 13: Antibacterial activity.

in the coprecipitation method may be the reason for its highest solubility. All the complexes could achieve improvement in wettability of Embelin due to the high aqueous solubility.[29-39]

The bioavailability of Embelin is 30±11%. A toxicology study of Embelin on experimental rats reported that it is safe at a dose of 20 mg/kg body weight/day for 30 days.[40] Enhancement of solubility and bioavailability by complexation can further reduce the dose of Embelin in its final formulation. So oral formulations based on complexes of Embelin are expected to have acceptable size.The antibacterial activity of pure Embelin and inclusion complexes was tested by using the viable cell-counting method. The inclusion complexes prepared from three methods were tested against two bacteria *E. coli* and *S. aureus*. Embelin shows significant antibacterial activity.[41] Antimicrobial activity of Embelin, physical mixture and complexes were high on Gram-positive organisms in comparison to that on Gram-negative organisms. This is consistent with the literature reporting MIC of Embelin for *S. aureus* and *E. coli* is 20µg/ml and 45µg/ml respectively.[6] This may be due to the presence of outer membrane in Gram-negative bacteria, the reason for their resistance to most antimicrobial agents.[42-44] In comparison to Embelin, the antibacterial activity of complexes by physical method, kneading and co-precipitation method was 30±0.2%, 47±0.1% and 96±0.01% for *E. coli* and 52±0.07%, 87±0.2% and 97±0.02% for and *S. aureus*. Complexes prepared by the kneading method showed a 1 log reduction in the number of *S. aureus* in comparison with EMB. But there was only a slight decrease in the number of *E. coli* as compared with EMB. Complexes prepared by the coprecipitation method showed 2 log reductions in the number of *S. aureus* as compared to EMB whereas 1 log reduction in the number of *E. coli* (Figure 13). This may be due to the formation of the inclusion complex in the right proportions in the co-precipitation method. This may be due to the formation of the inclusion complex in the right proportions in the co-precipitation method. In the co-precipitation method, inclusion complexes were formed at the molecular level as this method involves heat and stirring assisted bombardment of molecules with adequate energy.[45] This can be interpreted in the light of literature that reports quinones having pharmacophores for antibacterial activities have functioned as substrates of bacterial efflux pumps.[46] So usefulness of Embelin complexes for combining with other efflux pump inhibitors in the fight against antimicrobial resistance in bacterial infections has to be investigated. Further studies on molecular mechanisms of antimicrobial activity of Embelin complexes have to be carried out.

CONCLUSION

Phase solubility studies showed that stoichiometric ratio complexes of Embelin and β-cyclodextrin are 1:2. Inclusion complexes of Embelin were prepared by physical mixture, kneading and co-precipitation method. Complex formation was confirmed by FT-IR, DSC and SEM. Complexes prepared by the co-precipitation method showed fast and highest Embelin solubility. Antimicrobial studies confirmed that complexes prepared by kneading and co-precipitation methods showed more sensitivity to Gram-positive microorganisms. Complexes prepared from the co-precipitation method showed 100- and 10-times reduction in the number of Gram-positive

microorganisms and Gram-negative microorganisms respectively in comparison to Embelin. So, Embelin β- cyclodextrin complexes have the potential for the development of Embelin dosage forms with enhanced biological activities.

ACKNOWLEDGEMENT

The authors are grateful to KSCSTE, Trivandrum for financial support by Student Project Grant.

ABBREVIATIONS

g/ml: Gram per milliliter; **mg/ml:** Milligram per milliliter; **°C:** Degree centigrade; **h:** hours; **min:** minutes; α: Alpha; **β:** Beta; γ: Gamma; v/v: Volume by volume; **ml:** Milliliter; **mM/ml:** Milli mol per milliliter; **µm:** Micrometer; **rpm:** Revolutions per minute; **min:** Minute; **nm:** Nanometer; **cm:** Centimeter; **mg:** Milligram; **kV:** Kilovolt; **CFU/ml:** Colony-forming unit per milliliter; **CD:** Cyclodextrin; **log:** Logarithm.

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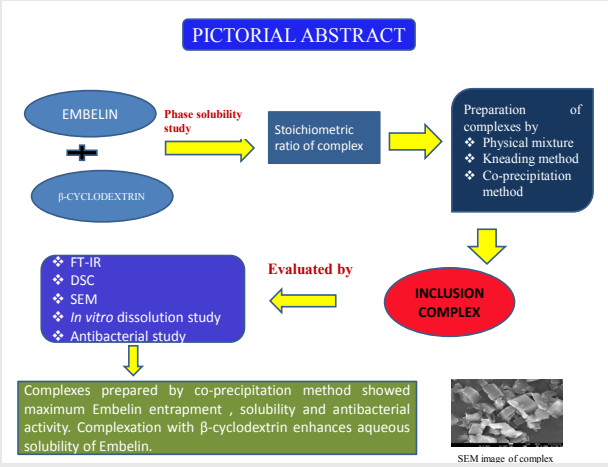
PICTORIAL ABSTRACT



SUMMARY

The present study aims to improve the water solubility and rate of dissolution of Embelin by the preparation of inclusion complexes with β-cyclodextrin. Inclusion complexes were prepared by kneading and co-precipitation methods. Fourier-Transform Infrared (FT-IR) spectroscopy and *in vitro* dissolution study were used to characterize the complexes. Complexes prepared by the co-precipitation method were also analyzed by Differential scanning calorimetry (DSC) and Scanning electron microscopy (SEM). Antimicrobial studies of complexes against *Staphylococcus aureus* and *Escherichia coli* were carried out by colony counting method. Phase solubility study showed Embelin forms complex with β-cyclodextrin in the ratio 1:2. FT-IR, DSC and SEM confirmed the formation of a complex of Embelin with β-cyclodextrin. Complexes of Embelin prepared by co-precipitation method resulted in the largest percent drug content, enhanced aqueous solubility and antibacterial activity.

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Cite this article: Kani JK, Xavier EN. Solubility Enhancement of Embelin by Complexation with Beta Cyclodextrin. Indian J of Pharmaceutical Education and Research. 2021;55(2s):s405-s413.